

**Original Research Article****Quantitative reckoning of embelin from fruits of *Embelia tsjeriam-cottam* using water bath process as an alternate method of extraction**

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ABSTRACT

The world is culturally endowed with various forms of healing practices having rich medical wisdom of immense importance. One of such pharmacognostically indispensable medicinal plant is *Embelia tsjeriam-cottam* A. DC. The active principle of *E. tsjeriam-cottam* is embelin, possessing a range of pharmacognostic activities including anti-cancer, antioxidant, antiinflammation, antibacterial and analgesic effects. Embelin in fruits of *E. tsjeriam-cottam* was extracted using water bath method. For extraction of embelin from fruits of *E. tsjeriam-cottam*, methanol and chloroform were used as solvents. Fruits were collected from five different agro-climatic zones of Odisha. Comparative estimation of embelin was done through spectrophotometer and High Performance Liquid Chromatographic (HPLC) methods. Samples extracted with chloroform and methanol showed embelin content in a range of 2.13-0.29% dry wt., and 0.95-0.28% dry wt. respectively using spectrophotometer. In case of HPLC analysis, samples extracted in chloroform and methanol showed embelin content in a range of 1.86-0.27% dry wt., and 0.875-0.26% dry wt. respectively. However the water bath method, used as alternative method for extraction, proved to be less time consuming, cost-effective in extracting a pretty good amount of embelin as compared to the conventional (soxhlet) methods of extraction.

Introduction

Use of medicinal plants has been known for centuries in many parts of the world for the treatment of various human ailments. The far-flung use of herbal formulations obtained from natural compounds for healthcare system has been accelerated in recent years. Pharmacological screening of compounds of natural or synthetic origin has been the source of inestimable therapeutic agents [1]. Embelin ($C_{17}H_{26}O_4$) is a naturally occurring phenolic compound in the benzoquinone group mostly found in the plants of Myrsinaceae family [2, 3] and is the active principle found in the fruits of *Embelia tsjeriam-cottam* [4-6].

E. tsjeriam-cottam, under vulnerable category [7], is commonly known as baibidanga and is highly esteemed in Ayurvedic system as a potent medicinal plant [8]. Embelin elicits diverse pharmacological activities like anticancer [8],

antioxidant [9], analgesic [10], anti-inflammatory [10], antiproliferative [11], hepatoprotective [12], antimicrobial [13], antidiabetic [14], wound healing [15], cardio-protective properties [8] and many more. Some of the vital medicinal properties of embelin are described below in [Table-1 & Figure-1]. Due to its diverse ethno-medicinal and pharmacological activities, its quantitative assessment from various sources required to be done following cost-effective and yield-oriented methods. Recently, our laboratory had assessed embelin from fruits of *E. tsjeriam-cottam* collected from various agro-climatic zones using Soxhlet method [16]. It is a very popular process of extraction where the solvents got mixed to the powdered samples in a drop wise manner for extracting the bio-active compounds at a controlled temperature system. However in case of water bath method, the powdered samples are completely exposed i.e. mixed thoroughly with the given solvent system at a particular temperature. Hence this simple, cost effective and efficient

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method of extraction can be implemented for extracting the bio active compounds in a huge amount as compared to the Soxhlet method. This research paper deals with the

quantitative assessment of embelin content in the fruit of *E. tsjeriam-cottam*, collected from different agro-climatic zones of Odisha using water bath system of extraction.

Table-1: Biological Activities of Embelin

Biological Activity of Embelin	References
Cytotoxicity Activity	[2]
Antioxidant Activity	[9, 10, 36, 37]
Anti-inflammatory Activity	[10, 32]
Antiproliferative Activity	[11, 50]
Wound healing Activity	[15]
Anthelmintic Activity	[38, 39]
Analgesic Activity	[10, 40]
Anticancer Activity	[41, 42]
Antimicrobial Activity	[5, 43]
Hepatoprotective Activity	[12, 48]
Antidiabetic Activity	[14, 49]
Antipyretic Activity	[44]
Strengthens the nervous system	[47]
Contraceptive Activity	[45, 46, 51]

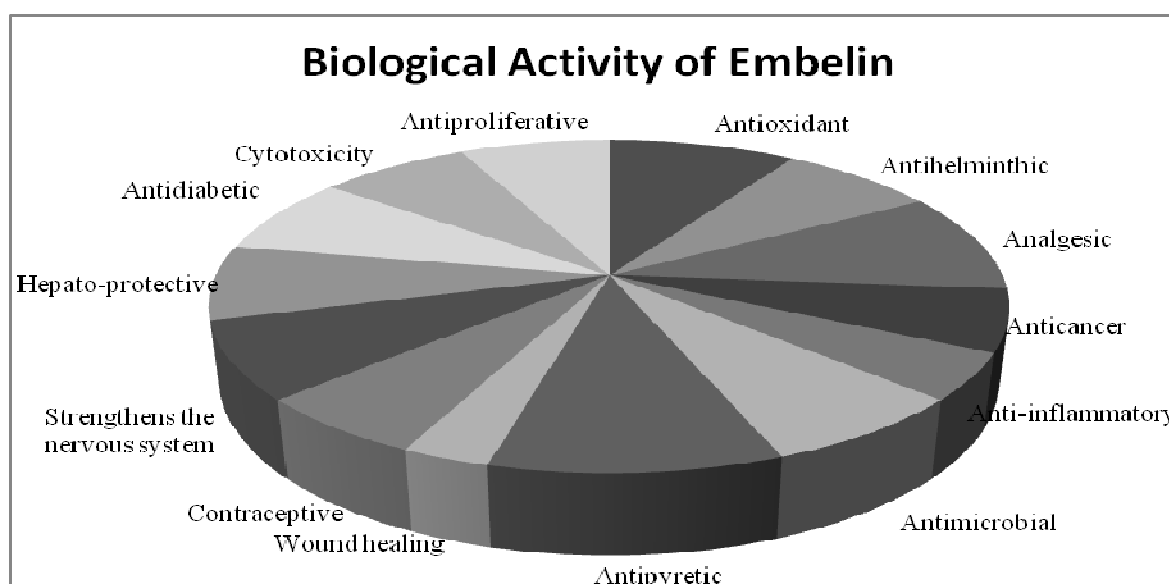


Figure 1: Biological Activities of Embelin

Materials & Methods

Materials

Fruit samples of *Embelia tsjeriam-cottam* were collected from various agro-climatic zones Odisha namely Bargarh (F1), Jajpur (F2), Chura Reserve Forest (F3), Sulia Reserve Forest (F4) and Ghana Reserve Forest (F5). All the source plants were authenticated by comparing with herbarium specimens present in the institutional herbarium (4897) and also verified through the reference book The Flora of Odisha [17].

Methods

Standard Preparation

For identification of the desired compound in sample extract, standard stock solution was prepared with synthetic Embelin (SIGMA Aldrich) in suitable solvent (Methanol) and kept in dark at 4°C for further use.

Extraction of Sample

Fruit samples were first powdered finely before using for extraction in water bath. Powdered samples (5 gm each) were

extracted at 60°C through water bath for a period of 18-20 hrs using Methanol and Chloroform solvent systems separately [18]. The filtrate was collected and the residue was again extracted through same procedure. This method was repeated consecutively for three times and all the collected filtrates were condensed using dry bath and kept as Embelin stock sample [19].

Assessment through UV-VIS Spectrophotometer

For assessing embelin content of all the extracts using spectrophotometer, a standard calibration curve was prepared in the first phase. For this purpose, the standard stock solution of increasing concentrations (ranging from 100-1000 µg/ml) of embelin standard was prepared by serial dilution method. A standard calibration curve was prepared. The absorbance of the prepared standard solution was measured at 291 nm wavelength and the calibration curve was plotted between the measured absorbance and given concentrations. The test sample extracts were also measured in the same wavelength and the embelin content was quantified [20, 21].

Identification & Isolation through Thin Layer Chromatography

The fruit samples extracted through water bath system were analyzed through thin layer chromatographic method for identification followed by isolation of the pure Embelin compound. For separation of pure embelin compound from the crude extracts, the previously standardised mobile phase (n-Propanol: n-Butanol: Ammonia in a ratio of 7: 1: 2) was selected [16]. The identified bands of embelin were detected under ultraviolet light at 365 nm wavelength and for naked eye observation these were treated with chromatographic reagent containing 1% solution of vanillin in methanolic sulfuric acid. For isolation of the compounds, the R_f value of each samples was determined and compared against the standard embelin solution [18, 22, 23].

Assessment through High Performance Liquid Chromatography

For quantitative assessment of the isolated compounds, HPLC analysis was performed in a Waters make HPLC system

equipped with a binary pump (Model-1525) and porous Silica with 5 µm diameter C 18 4.6 × 150 mm column. The isolated samples were analysed through HPLC system using a mixture of Methanol: 0.1 % TFA (in HPLC grade water) in a ratio of 88:12, at a flow rate of 1 ml/min [24-26]. All the samples (20 µl) were injected to the pump individually and the analysis was carried out for a time period of 8 minutes. The peaks eluted were detected at 291 nm wavelengths and compared with the authentic standard Embelin solution for identification. For accuracy in the results, the standard embelin was estimated with ten replicate injections and all the samples with three replicate injections. The HPLC process used for the estimation was validated by checking the linearity, peak purity, retention time, co-relation coefficient, limit of quantification and detection, relative standard deviation, accuracy and specificity [27]. The chromatograms are given in [Figure-5 & 6].

Statistical Analysis

All the result values are expressed as Mean ± SD (n=3). Data were analyzed statistically through Two-way RM ANOVA followed by Sidak's multiple comparisons test using GRAPH PAD PRISM 6.0 and variations in both Spectrophotometric and HPLC results were observed at 99% significant level.

Results

Assessment of Embelin Content through spectrophotometer

Embelin content was found to be ranged from 0.28-2.13% dry wt. in the wild fruits collected from different agro-climatic zones. Chloroform extracted fruit samples collected from F3 region yielded maximum embelin content (2.13% dry wt.) whereas fruit sample from F2 gave the minimum amount of embelin (0.29% dry wt.). The results showed significant variation at P value < 0.0001. Methanol extracted fruit samples collected from F3 region produced maximum embelin (0.95% dry wt.) and fruit samples from F2 yielded minimum amount of embelin (0.28% dry wt.). The results showed significant variation at P value = 0.0014 [Table-2, Figure-2].

Table 2: Embelin content (% Dry Wt.) in various fruits of *Embelia tsjeriam-cottam* estimated through Spectrophotometer

Solvents Used for Extraction	Region	% of Embelin Content
Methanol	F1	0.78 ± 0.354 **
	F2	0.28 ± 0.183
	F3	0.95 ± 0.191****
	F4	0.902 ± 0.185 ns

Chloroform	F5	0.625 ± 0.205
	F1	1.68 ± 0.08
	F2	0.29 ± 0.035 ns
	F3	2.13 ± 0.496
	F4	2.1 ± 0.0702 **
	F5	1.5 ± 0.106

Abbreviations-F1-Bargarh, F2-Jajpur, F3-Chura Reserve Forest, F4-Sulia Reserve Forest, F5-Ghana Reserve Forest

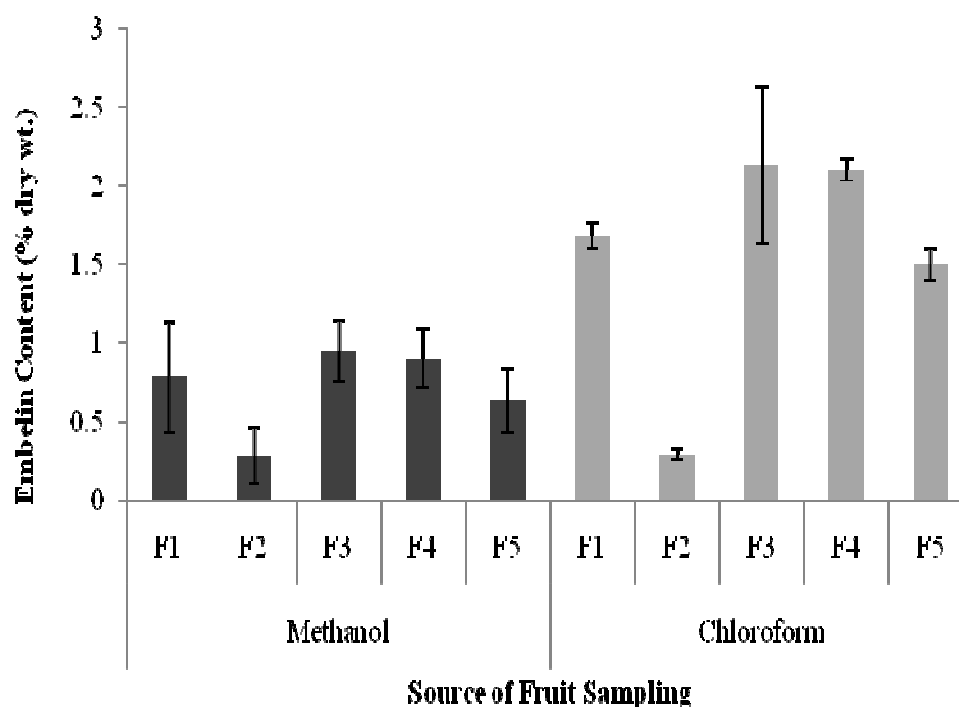


Figure 2:Embelin content (% Dry Wt.) in various fruits of *Embelia tsjeriam-cottam* estimated through Spectrophotometer

Abbreviations-F1-Bargarh, F2-Jajpur, F3-Chura Reserve Forest, F4-Sulia Reserve Forest, F5-Ghana Reserve Forest

NB-All values expressed as Mean ± SD (n=3). The statistical differences were tested by Two-way RM ANOVA followed by Sidak's multiple comparisons test, where ****P<0.0001, **P=0.0014 and ns=Not Significant

Assessment of Embelin Content through HPLC

During the course of isolation of pure Embelin for HPLC, the R_f values of the standard and the extracted samples were found to be 0.35 through TLC [Figure-3]. Amongst Chloroform extracted fruit samples, the highest amount of Embelin was found in the samples belonging to F3 (1.86% dry wt.) whereas fruit samples from F2 yielded the minimum

amount of embelin (0.27% dry wt.). The results showed significant variation at P value < 0.0001. However Methanol extracted fruit samples collected from F3 produced the maximum embelin (0.875% dry wt.) and fruit samples from F2 showed minimum amount of embelin content (0.26% dry wt.). The results showed significant variation at P value = 0.0405 [Table-3, Figure-4].

Table 3: Embelin content (% Dry Wt.) in various fruits of *Embelia tsjeriam-cottam* estimated through HPLC

Solvents Used for Extraction	Region	% of Embelin Content
Methanol	F1	0.737 ± 0.040 *
	F2	0.26 ± 0.026
	F3	0.875 ± 0.156 ****
	F4	0.75 ± 0.134 ns
	F5	0.42 ± 0.043
Chloroform	F1	1.28 ± 0.456
	F2	0.27 ± 0.026 ns
	F3	1.86 ± 0.476
	F4	1.66 ± 0.036 *
	F5	1.19 ± 0.234

Abbreviations-F1-Bargarh, F2-Jajpur, F3-Chura Reserve Forest, F4-Sulia Reserve Forest, F5-Ghana Reserve Forest

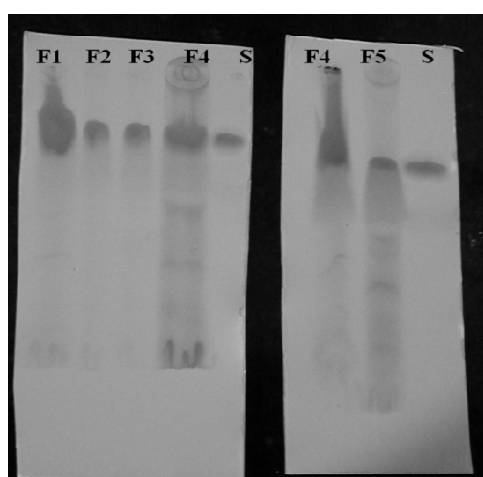


Figure 3: Presence of Embelin in Sample Extracts against Standard on TLC Sheets

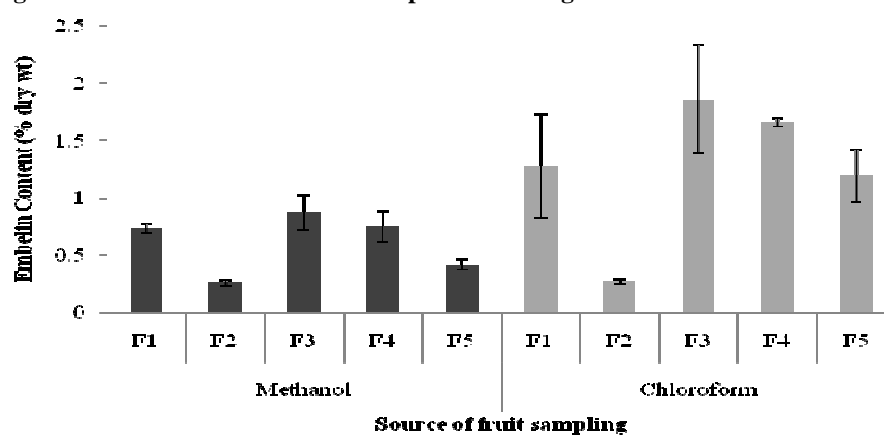


Figure 4: Embelin content in various fruits of *Embelia tsjeriam-cottam* estimated through HPLC

Abbreviations-F1-Bargarh, F2-Jajpur, F3-Chura Reserve Forest, F4-Sulia Reserve Forest, F5-Ghana Reserve Forest

NB-All values expressed as Mean \pm SD (n=3). The statistical differences were tested by Two-way RM ANOVA followed by Sidak's multiple comparisons test, where ***P<0.0001, *P=0.0405, and ns=Not Significant

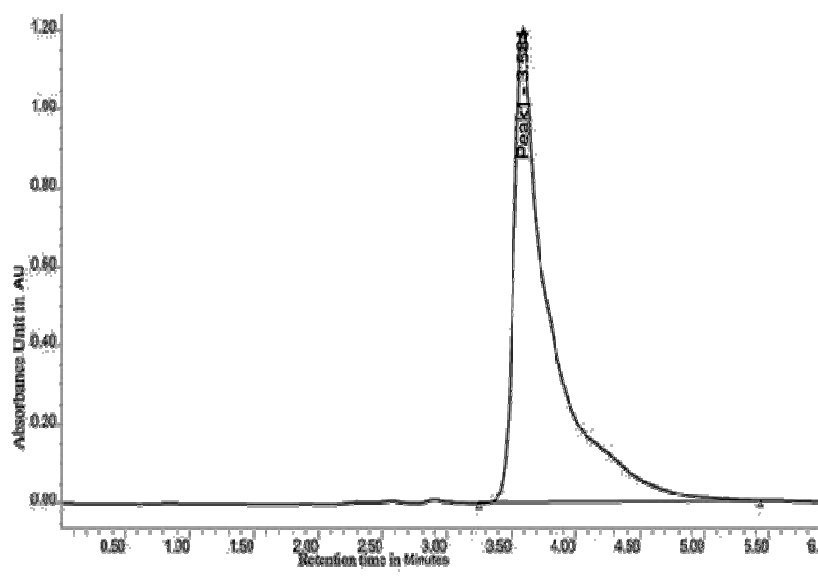


Figure 5:HPLC Chromatograms of Embelin Standard

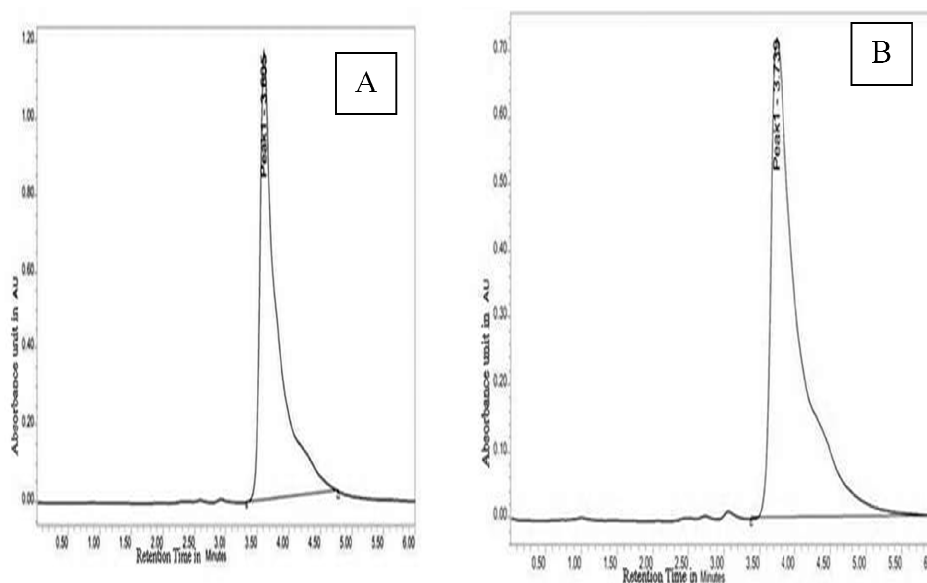


Figure 6:HPLC Chromatograms of embelin (A) Methanol extracted Embelin Samples and (B) Chloroform extracted Embelin Samples

Accuracy/Recovery Test of Embelin

To check the accuracy of the developed method and to study the interference of samples, recovery experiment was carried out by standard addition method [Table-4]. A known amount of sample was taken. To each tube known amount of Embelin

was added. Each sample was analyzed by the developed HPLC method and the amount of Embelin recovered for each level, was calculated.

Table 4: Recovery/Accuracy Test of Embelin through HPLC Analysis

Sl No.	In Sample (mg/ml)	Added (mg/ml)	Estimated (mg/ml)	% RSD	% of Recovery
1	50	100	146.37±0.007	0.0048	97.58
2	100	150	247.72±0.061	0.025	99.1
3	150	200	347.65±0.032	0.009	99.33

NB-All values expressed as Mean ± SD, (n=3)

Discussion

Among all quinones, benzoquinone and its analogues are most widely used to elicit pharmacological action. Embelin is one of the biologically active benzoquinone derivatives that act as the active principle compound in the fruits of *Embelia tsjeriam-cottam* and responsible for its medicinal properties[5]. Several literatures are available regarding the extraction of embelin from fruits of *E tsjeriam-cottam* and also *E ribes* using methanol and/or chloroform as solvent system separately [4, 6, 18, 19, 24, 28-30]. However as far as the comparison between the selected solvent systems is considered in our study, chloroform showed promising result in extraction process of embelin than the Methanol and this observation was also validated by our previous work [16].

Mostly the extraction of embelin from fruits of *E tsjeriam-cottam* and its close associate *E ribes* were done through conventional Soxhlet method [16, 19, 20, 27, 29, 31-33]. However several alternative methods regarding the extraction of embelin are also available. Some of them are maceration method [28, 34], water bath method [4, 18, 23], microwave-assisted method [35]. Yield of embelin from alternative methods instead of the conventional method showed remarkable variations. However in this case we have targeted the water bath process of extraction for yielding the embelin from fruits of *E tsjeriam-cottam*, collected from five different agro-climatic zones of Odisha. In an investigation, the fruits of *Embelia ribes* were extracted through water bath extraction process and quantitative analysis (4.8% w/w) was done through Spectrophotometer [23]. According to another finding, the embelin content from fruits of both *E ribes* and *E tsjeriam-cottam*, extracted through water bath process, was found to be 4.33% and 3.96% w/w respectively [4]. Similarly in another case, the fruits of *E ribes* were also extracted with methanol through water bath system [18].

In our previous study, embelin content in the fruits of *E tsjeriam-cottam*, collected from various agro-climatic zones of Odisha through Soxhlet method of extraction, ranged in between 1.03-4.93% dry wt. in case of spectrophotometric method and 0.5-2.72% dry wt. in case of HPLC analysis [16]. However, in the present study, using water bath method, the embelin content in the fruits of *E tsjeriam-cottam*, collected

from various agro-climatic zones of Odisha, was estimated to be in a range of 0.28-2.13% dry wt. in case of spectrophotometric method and 0.26-1.86% dry wt. in case of HPLC analysis. An attempt was made to isolate and quantify embelin from fruits of *E tsjeriam-cottam*, collected from five different regions for comparative assessment of zonal differentiation through HPLC analysis. Range of embelin content in all the mentioned agro-climatic zones are found to be in the same pattern as we found in our previous study i.e. the highest amount of embelin content was found in Chura Reserve Forest (F3) followed by Sulia Reserve Forest (F4), Bargarh (F1), Ghana Reserve Forest (F5) and finally by Jajpur (F2). Lower but remarkable quantity of embelin was found in the fruit samples when extracted with a new method of extraction.

Conclusion

Embelia tsjeriam-cottam, as an alternative source of embelin to that of very rare and endangered *E. ribes*, was evaluated for estimation of embelin content both through Spectrophotometric and HPLC methods. Crude embelin was extracted from the fruits, collected from various agro-climatic zones of Odisha, through water bath process of extraction. In this study, *E tsjeriam-cottam* fruits, collected from Chura reserve Forest of Odisha is prevailed to be the best source for embelin with more embelin content when extracted following 'water bath method' as compared to the conventional soxhlet method. Though the conventional Soxhlet method is an excellent method for the yield of higher quantity of embelin in pure form, the water bath method in this study was found to be superior to Soxhlet method in terms of consumption of less time duration and less amount of samples and solvents for extraction along with high yield rate in terms of crude extracts. This method can be applicable for extracting a huge amount of samples in short time span.

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Conflict of interest: We declare that we have no conflict of interest.

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